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Mutations in the tumour suppressor breast cancer susceptibility gene 2 (BRCA2), identified in 1994 using linkage analysis and cloned in 1995, are associated with 30-40% of all familial breast cancer cases and, to a lesser extent, with ovarian and pancreatic cancer. In addition, mutations in BRCA2 are strongly linked to hereditary breast cancer in males. Most research has focussed on BRCA2 and its involvement in DNA repair due to cellular sensitivity to DNA damaging agents in BRCA2-null mice. Less studied are other possible functions that BRCA2 may perform. Using BRCA2 in a yeast two hyrbid assay we identified tristetraprolin (TTP) as a potential interacting protein. TTP was previously shown to be involved in the degradation of TNF-α mRNA. This was underscored by TTP-null mice displaying high levels of TNF-α and the manifestation of autoimmune-like complications as a result of these elevated levels. We have mapped the area of interaction on BRCA2 using an interaction mating analysis to a stretch of ~80 amino acids. We have also demonstrated an *in vivo* interaction in transfected 293 cells between BRCA2 and TTP. Future studies will attempt to ascertain whether this interaction exerts an influence on the levels of TNF-α mRNA.

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Introduction

Breast cancer is one of the most prevalent human cancers and a leading cause of death among women. Germline mutations in the breast cancer susceptibility gene 2 (BRCA2) are associated with 30-40% of hereditary breast cancer cases and recently, biallelic BRCA2 mutations were shown to be responsible for complementation group D of Fanconi Anemia[1,2]. Most of the research has focussed on BRCA2 and its involvement in DNA repair due to cellular sensitivity to DNA damaging agents in BRCA2-null mice[3,4]. The association of BRCA2 with DNA has also been explored involving studies with RAD51 and DSS1, proteins shown to interact not only with BRCA2 and DNA but, as well, in combination[5,6]. Less well known is the involvement of BRCA2 in other cellular processes besides maintenance of genomic integrity. To address this possibility we assayed a large portion of the BRCA2 protein in a yeast two hybrid system. The use of a human and murine mammary cDNA libraries yielded, among others, three independent clones coding for human tristetraprolin gene (TTP) and seven independent clones coding for the murine Limd1 gene. TTP was previously shown to be involved in the degradation of TNF-α mRNA[7]. This was underscored by TTP-null mice displaying high levels of TNF-α and the manifestation of autoimmune-like complications as a result of these elevated levels. These symptoms were alleviated by the injection of antibodies towards the TNF-α. The Limd1 gene is of interest due to its location in a region that exhibits high levels of LOH in a number of cancers[8].

The purpose of this proposal was to validate the proposed interaction of BRCA2 and TTP *in vivo* and, if direct, *in vitro* and to further physically map the location of this association. To be addressed in the coming year is the possible effects that this association, or lack thereof, would have on the levels of TNF-α.

Body

Year 1

Task 1. The confirmation of the BRCA2/TTP protein interaction using in vitro and in vivo model systems and to further define the minimal region responsible for interaction (1-12 months).

The original BRCA2 bait used in the yeast two-hybrid was large in size at approximately 140 kDa (Figure 1). Since our hybrid system had already demonstrated a specific interaction between BRCA2 and TTP, we utilized our established hybrid assay to map the region in the original bait responsible for the interaction. This was accomplished by generating multiple constructs of the original bait for use in a simple interaction mating analysis with one of the full-length TTP clones (Figure 2a). The initial set of mating analysis indicated that most of the positive clones from the hybrid were interacting with the first portion (F-3-1, Figure 2a) of the original bait. This prompted further deconstruction of the BRCA2 F-3-1 bait in order to determine whether there was a

"sticky" portion of this particular bait. Figure 2b demonstrated TTP interacted with a specific portion of the BRCA2 (~80 a.a.), separate from other potential positive clones indicating a distinct domain responsible for the association.

To address the interaction in mammalian cells, HEK 293 cells were transfected with full-length BRCA2 and TTP-FLAG vectors. Using these transfected cells we have shown one-way Western analysis of immunoprecipitated BRCA2 demonstrated an *in vivo* interaction (Figure 3). The results also suggest that TTP interacts with BRCA2 in its multiple phosphorylated states. These different levels of phosphorylation are speculated to be associated with its ability to degrade TNF-α mRNA[9]. The reverse co-immunoprecipitation (immunoprecipitating for TTP-FLAG and blotting BRCA2) has proven difficult given the large size of BRCA2 (400 kDa). The technical difficulties of this aspect continued to be addressed. We have also acquired aliquots of a polyclonal anti-human TTP antibody that came to our attention from recently published work. Unfortunately, this antibody is unable to detect endogenous levels of TTP and prevents this type of analysis at this point. We did, however, use the antibody in the same manner as the FLAG antibody (under transfected conditions) and observed similar results (Figure 3b). Attempts have been made to transfect BRCA2 and TTP-FLAG into cell lines MCF-7 and Capan-1 for further in vivo analysis. To date this has proven elusive given the inherent difficulty in transiently transfecting these particular lines, but we are currently generating stable transfectants in these cells lines.

Key Research Accomplishments

- A proposed interaction between BRCA2 and TTP emanating from a yeast two hybrid assay using a human mammary cDNA library.
- Fine-mapping region within BRCA2 responsible for the association with TTP to a stretch of 80 amino acids (from an original 1300 amino acid bait).
- *In vivo* coimmunoprecipitation of TTP-FLAG in transfected HEK 293 cells.

Reportable Outcomes

2002 AACR Annual General Meeting. San Francisco, CA.
Huggins, C.J. and Andrulis I.L. Characterization of a proposed novel BRCA2 interaction

2003 AACR Annual General Meeting. Toronto, ON.
Huggins, C.J. and Andrulis I.L. Functional and genetic characterization of the LIMD1 gene.

Conclusions

An important accomplishment to date is the fine mapping of the region within BRCA2 responsible for the association with TTP. The large size of the original bait (1,300 a.a.) would most likely hinder further biochemical work with these two proteins, but the generation of a shorter BRCA2 region makes future work more manageable. According to the Human Genome Database there are a number of breast cancer associated mutations found within this stretch of sequence. It is conceivable that the association of TTP with BRCA2 and ultimately, levels of TNF-α could be affected by these particular mutations, a possibility we are going to address by generating BRCA2 mutants.

We have also shown a one-way *in vivo* association between BRCA2 and TTP under transfected conditions. We are currently working on demonstrating the interaction in the reverse direction.

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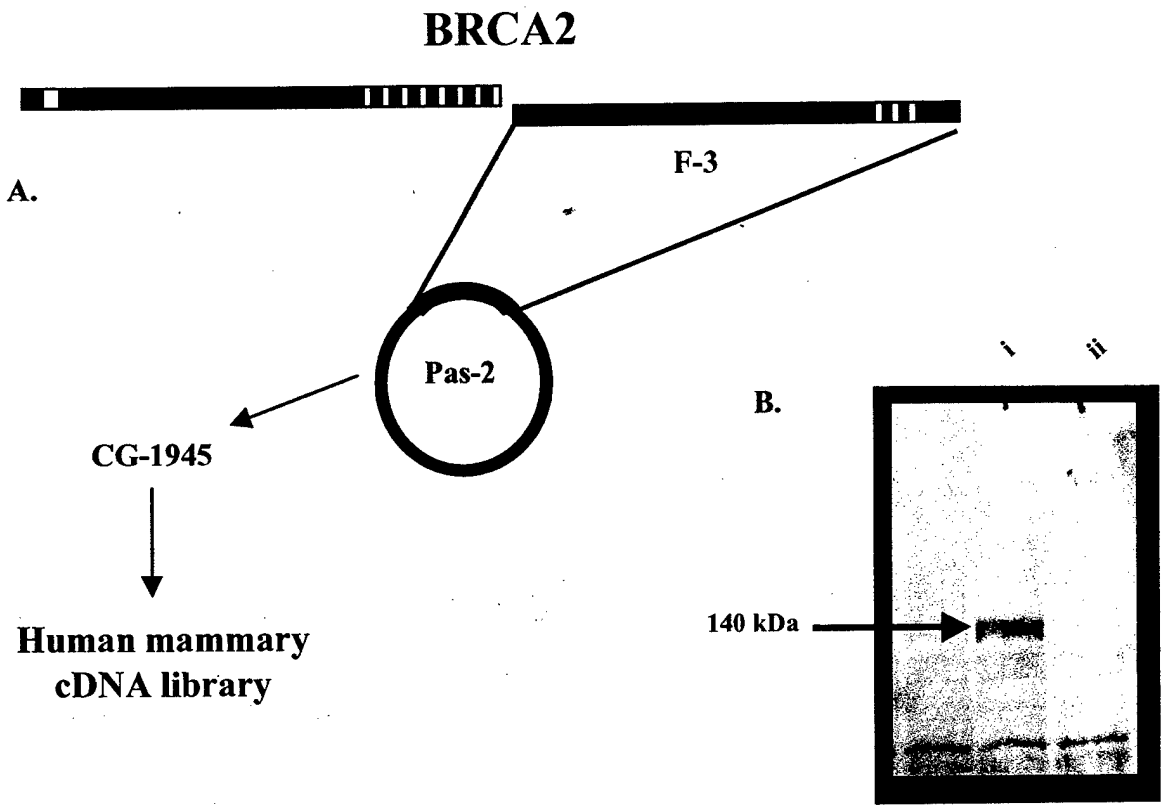


Figure 1. A. Fragment 3 (F-3, amino acids 2116-3418) was ligated into the Pas-2 (F-3 Pas-2) DNA-binding domain vector, transfected into strain yeast strain CG-1945 and assayed for interacting proteins using a human mammary cDNA library. B. Western blot using DNA-binding domain antibody demonstrating expression of fragment 3 (i) versus empty Pas-2 vector (ii) in CG-1945.

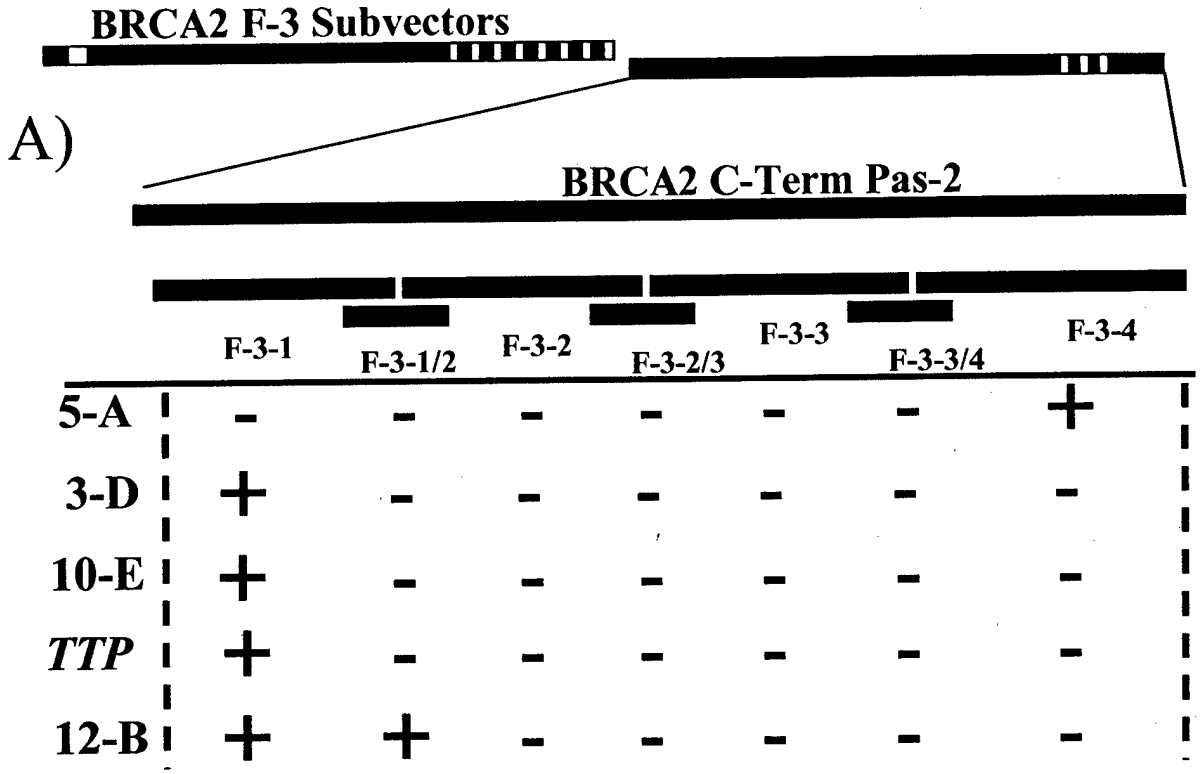
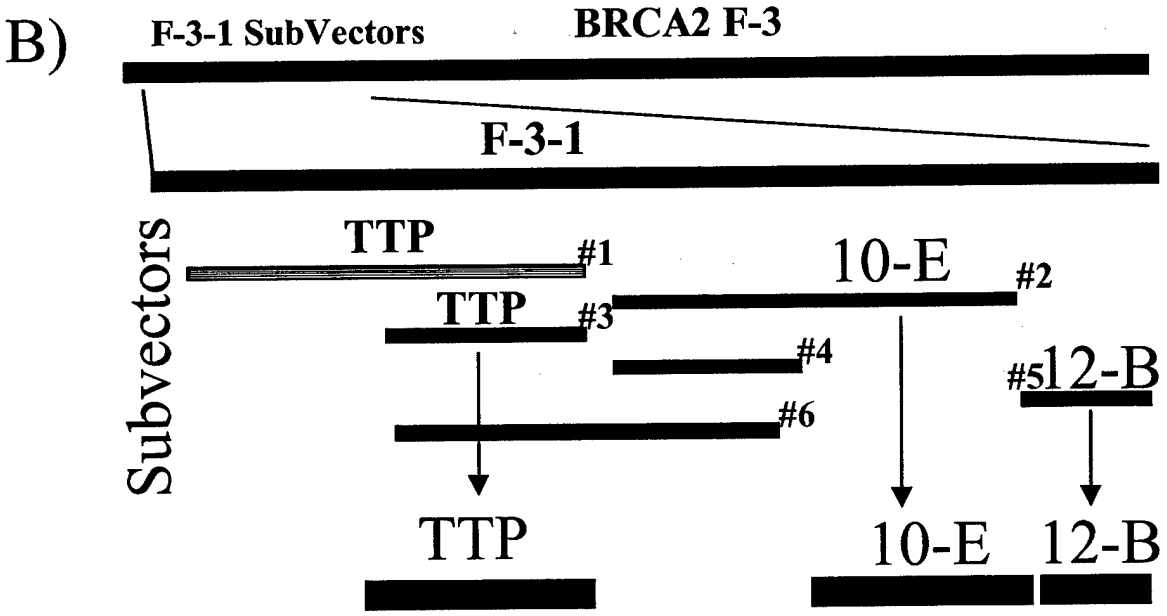


Figure 2. A) Interaction mating analysis of a divided original BRCA2 F-3 bait. Yeast strain Y187 was transformed with BRCA2 bait plasmids while strain CG-1945 was transformed with potential interactors. Overnight matings were plated on dropout media to assay for interactions. The majority of the potential positive interactions were associating with BRCA2 F-3-1, the first portion of the bait. B) Further subdivision of the BRCA2 F-3-1demonstrated distinct areas of interaction within this bait



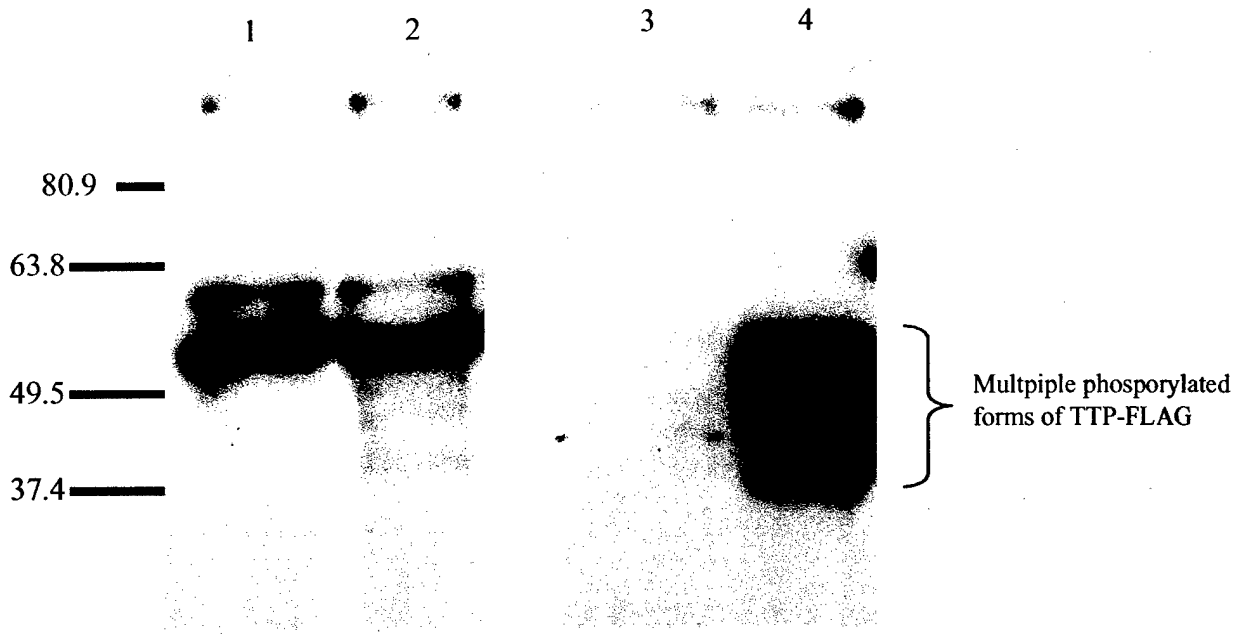


Figure 3 A) Coimmunoprecipitation of transfected TTP-FLAG with BRCA2 in 100 mm plates of HEK 293 cells. Both lanes 1 and 2 were transfected with BRCA2 while lane 1 was transfected with empty FLAG vetor and lane 2 with TTP-FLAG. Both were immunoprecipitated with BRCA2 (Ab-1) antibody. Western analysis was carried out with anti-FLAG antibody. Lanes 3 and 4 are the lysate.
B) Coimmunoprecipitation of transfected TTP-FLAG with BRCA2 in 100 mm plates of HEK 293 cells Lanes 1-4 are transfected increasing concentrations of BRCA2 while lane 1 represent empty FLAG vector. Western analysis was carried out using polyclonal anti-TTP antibodies. Lanes 5-8 are lysates.